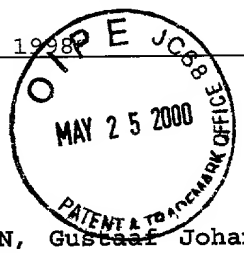
529 Rec'd PCT/PTO 25 MAY 2000

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		Attorney's Docket Number 01975.0024
		U.S. Application No. 09/555139 PCT
International Application. No.	International Filing Date	Priority Date Claimed
PCT/EP98/07553	November 24, 1998	November 25, 1997

Title of Invention:
VACCINES WITH AN LTB ADJUVANT



Applicant(s) For DO/EO/US:
Etienne AGSTERIBBE, Rudi BRANDS, Lolke DE HAAN, Gustaaf Johan Marie VAN SCHARRENBURG, Willem Ronald VERWEIJ and Jan Christiaan WILSCHUT

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [X] Annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [ ] Other items or information:
    a. [ ] Verified Small Entity Statement.
    b. [ ] Copy of Notification of Missing Requirements.

U.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NUMBER
09/555139	PCT/EP98/07553	01975.0024

17. [X] The following fees are submitted: | CALCULATIONS

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO.....$840.00

International preliminary examination fee paid to USPTO (37 CFR 1.482).....$670.00

No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....$690.00

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....$970.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....$ 96.00

ENTER APPROPRIATE BASIC FEE AMOUNT = $ 840.00

Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)). $

Claims	Number Filed	Number Extra	Rate	
Total Claims	17 -20=		X $18.00	$
Independent Claims	8 - 3=	5	X $78.00	$ 390.00
Multiple dependent claim(s) (if applicable)			+$260.00	$ 260.00
		TOTAL OF ABOVE CALCULATIONS =		**$1,490.00**
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28)				$
		SUBTOTAL =		**$1,490.00**
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$
		TOTAL NATIONAL FEE =		**$1,490.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31).		$40.00 per property	+	$
		TOTAL FEES ENCLOSED =		**$1,490.00**
		Amount to be refunded		$
		charged		$

a. [X] A check in the amount of **$1,490.00** to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-0916. A duplicate copy of this sheet is enclosed.

The Commissioner is hereby authorized to charge any other fees due under 37 C.F.R. §1.16 or §1.17 during the pendency of this application to our Deposit Account No. 06-0916.

SEND ALL CORRESPONDENCE TO:
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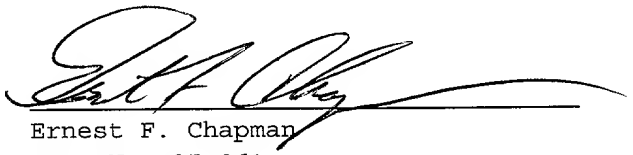

Ernest F. Chapman
Reg. No. 25,961

Submitted: May 25, 2000

PATENT
Attorney Docket No. 1975.0024-00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Etienne AGSTERIBBE et al.	
Serial No.: Unassigned	Group Art Unit: Unassigned
U.S. National Stage Application of: PCT/EP98/07553	Examiner: Unassigned
PCT Filed: November 24, 1998	
National Stage Entry: May 25, 2000	
For: VACCINES WITH AN LTB ADJUVANT	

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Prior to the examination of the above application, please amend this application as follows:

IN THE SPECIFICATION:

On page 5, line 22, delete "op" and replace with --of--.

On page 6, line 3, after "however," insert --it--.

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IN THE CLAIMS:

Without prejudice or disclaimer, please delete claims 1-9 and add claims 10-23 as follows:

--10. A vaccine composition comprising at least one particulate immunogen and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli,* wherein said B subunits are free of A subunit and toxic LT holotoxin.

11. The vaccine composition according to claim 10, wherein said B subunits are prepared by recombinant DNA methods.

12. The vaccine composition according to claim 10 or claim 11, wherein the immunogen comprises a viral antigen, a bacterial antigen, or a fungal antigen, or a combination thereof.

13. The vaccine composition according to claim 10, wherein the immunogen is derived from at least one infective agent which causes a disease which is transmitted by mucosal infection.

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14. The vaccine composition according to claim 10, wherein the immunogen is characteristic of a micro-organism which causes a disease which is transmitted by mucosal infection.

15. The vaccine composition according to claim 10 or claim 11, wherein the immunogen provides immunization against a disease which is transmitted by mucosal infection.

16. The vaccine composition according to claim 15, wherein the immunogen comprises influenza antigens.

17. A method for the induction of a systemic immunoglobulin response against an immunogen in a human or animal host in need of such induction, comprising the step of:

administering to mucosal tissue of the host said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli,* wherein said B subunits are free of A subunit and toxic LT holotoxin, and wherein said immunogen together with said B subunits is present in sufficient quantity for said induction.

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18. A method for the induction of a common mucosal immune response against an immunogen in a human or animal host in need of such induction, comprising the step of:

administering to mucosal tissue of the host said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli,* wherein said B subunits are free of A subunit and toxic LT holotoxin, and wherein said immunogen together with said B subunits is present in sufficient quantity for said induction.

19. A method of preparing a vaccine for the induction of a systemic immunoglobulin response against an immunogen in a human or animal host upon mucosal administration of said vaccine, comprising the step of:

combining said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli,* wherein said B subunits are free of A subunit and toxic LT holotoxin, and wherein said immunogen together with said B subunits is present in sufficient quantity for said induction.

20. A method of preparing a vaccine for the induction of a common mucosal immune response against an immunogen in a human or animal host upon local mucosal administration of said vaccine, comprising the step of:

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combining said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli,* wherein said B subunits are free of A subunit and toxic LT holotoxin, and wherein said immunogen together with said B subunits is present in sufficient quantity for said induction.

21. A vaccine comprising at least one particulate immunogen and an adjuvanting amount of B subunits of enterotoxin, wherein said B subunits are free of A subunit and toxic LT holotoxin.

22. A vaccine comprising at least one particulate immunogen and an adjuvanting amount of B subunits of cholera toxin, wherein said B subunits are free of A subunit and toxic CT holotoxin.

23. A vaccine comprising at least one immunogen and an adjuvanting amount of B subunits chosen from enterotoxin and cholera toxin, wherein said B subunits are free of A subunit, toxic LT holotoxin, and toxic CT holotoxin. --

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REMARKS

Two obvious typographical errors were corrected in the specification. One skilled in the art would recognize the existence of the errors, and the appropriate corrections. Thus, these corrections do not involve the introduction of new matter into the specification. *See* MPEP § 2163.07(II).

Claims 1-9 were deleted without prejudice or disclaimer, and new claims 10-23 were added to particularly point out and distinctly claim the subject matter which Applicants regard as their invention. Support for these amendments can be found in the specification and claims as originally filed. Care has been taken so that no new matter has been introduced into the application.

Applicants respectfully request examination and allowance of the amended claims. Applicants assert that the claims are in condition for allowance, and hope for an early favorable action on the merits.

If there is any fee due in connection with the filing of this Preliminary Amendment, please charge the fee to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

By: Carol P. Einaudi

Carol P. Einaudi
Reg. No. 32,220

Dated: May 25, 2000

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VACCINES WITH AN LTB ADJUVANT

The present invention relates to a vaccine containing the B subunits of heat-labile enterotoxin (LTB) of *Escherichia coli* (*E. coli*) as a mucosal immunoadjuvant. The invention relates in particular to a vaccine of this type to prevent influenza infections in humans. However, the invention is not restricted to application in influenza vaccines.

It is the object of vaccination against infectious diseases to prevent or at least restrain infection of the vaccinated subject by stimulating an immune response against the infectious agent through introduction of an antigen formulation derived from the particular pathogen. Ideally, the induced immune response should consist of two components, a humoral response (the production of antigen-specific antibodies) and a cellular response (the generation of specific cytotoxic T lymphocytes, capable of eliminating cells infected by the pathogen).

Many vaccination procedures involve the administration of a formulation containing inactivated or attenuated whole pathogen. However, for certain pathogens there is a considerable disadvantage to vaccination with whole pathogen, since such preparations, even though they are usually highly immunogenic, may have undesirable side effects. This explains the current trend towards the use of well-defined subunit vaccines or synthetic vaccines, substantially lacking the adverse side effects of the whole infectious agent. However, compared to whole pathogen, subunit vaccines or synthetic vaccines are often not very immunogenic, at least in the absence of an added adjuvant.

Adjuvants are substances or materials administered in conjunction with the antigen so as to stimulate the immune response against that antigen. There is a need for appropriate adjuvants which would boost the immune response against subunit antigens or synthetic antigens without causing undesirable side effects.

Influenza vaccine formulations have contained for a long time, and in some cases still contain, inactivated or attenuated whole virus. Such formulation may have considerable side effects, most notably fever and reactions at the site of injection. Nowadays,

vaccination is usually done with a subunit formulation. This subunit vaccine, which causes less side reactions, only contains the two major surface antigens of the virus, the hemagglutinin (HA) and the neuraminidase (NA), in a more or less purified form. In most current vaccine formulations there is no added adjuvant present.

The inactivated or attenuated whole influenza virus vaccine as well as the subunit vaccine are usually administered via a single intramuscular (i.m.) injection. The protection against influenza infection, achieved by either vaccination procedure, is comparatively low, particularly in elderly people. The relatively low efficacy of vaccination against influenza is due in part to the high antigenic variability of the virus. However, there is reason to believe that the protection against influenza infection by vaccination can be improved by stimulation and/or modification of the immune response against the antigen.

In the case of influenza, or in general in cases in which the infection is contracted via the respiratory tract, strategies for improved vaccination efficacy should aim at the generation of not only an adequate T-cell-dependent IgG response in the circulation, but also at a local immune response (secretory IgA) in the lungs and nasal cavity as a first line of defence against invading infectious virus. Furthermore, a cellular immune response (cytotoxic T-cells) might also be important, particularly in restricting the infection. It has been demonstrated that administration of influenza vaccine via i.m. injection (the current route of administration) does not result in a local IgA response in the respiratory tract.

The present invention relates to the surprising finding that the presence of LTB in an intranasal vaccine formulation not only stimulates the IgG response in the circulation, relative to i.m. immunisation with the adjuvant-free immunogen vaccine, but also generates a local IgA response in the respiratory tract.

The intact heat-labile enterotoxin (LT), and its close relative cholera toxin (CT), are composed of one A subunit and a pentameric ring structure consisting of five identical B subunits. The A subunit has enzymatic, ADP-ribosylation, activity and attributes the toxic activity to the toxins. In the intestinal epithelium the A subunit

induces persistent synthesis of second messenger cAMP, resulting in excessive electrolyte and concomitant fluid secretion to the lumen of the gut.

LT and CT are powerful mucosal immunogens. Upon local mucosal administration these molecules give rise to not only induction of a systemic antibody response directed against the toxin, but also to production of locally secreted antibodies, notably secretory IgA (S-IgA). LT and CT are also powerful mucosal immunoadjuvants. That is, when co-administered with an unrelated other immunogen, LT or CT may stimulate the systemic and mucosal antibody response against that immunogen. However, the toxicity of LT and CT has thusfar essentially precluded the use of LT or CT in human vaccine formulations.

In attempts to separate the toxic from the immune-stimulatory activities of LT or CT, detoxified mutants of the toxins, or the unmodified isolated pentameric B subunit (LTB or CTB, respectively), have been examined for their immunoadjuvant activity. Clearly, because the toxic ADP-ribosylation activity of the toxins resides in the A subunit, the presence of even trace amounts of unmodified A subunit or of LT or CT holotoxin in a human vaccine is highly undesirable.

The use of LTB as an adjuvant for influenza antigens has been investigated by Tamura and co-workers (Hirabashi et al.: Vaccine **8**: 243-248 [1990]; Kikuta et al.,: Vaccine **8**: 595-599 [1990]; Tamura et al. J.: Immunology **3**: 981-988 [1992]; Tamura et al.: Vaccine **12**: 419-426 [1994]; Tamura et al.: Vaccine **12**: 1083-1089 [1994]). In these studies, based on the use of soluble influenza virus hemagglutinin (HA) vaccine, extracted and purified from influenza virus by treatment with Tween/ether according to Davenport et al (J. Lab. & Clin. Med. **63**(1): 5-13 [1964], it was established that LTB, free of A subunit, lacks mucosal immunoadjuvant activity when administered intra-nasally in conjunction with the soluble HA antigen to mice. It was further demonstrated that the presence of trace amounts of holotoxin, for example residual holotoxin remaining in B subunit preparations isolated from holotoxin, restores the expression of adjuvant activity of LTB towards the soluble HA antigen. More in particular, when LTB from recombinant sources (and therefore, completely free of even the smallest trace amounts of A subunit) was used, a trace of holotoxin

had to be added in order for the LTB to exert mucosal activity upon intranasal co-administration with the soluble HA antigen.

Surprisingly, it was found that isolated LTB from recombinant origin and therefore completely free of A subunit, does possess powerful immunoadjuvant activity depending on the nature or presentation form of the intranasally co-administered immunogen.

For example, adjuvant activity towards freely mixed small soluble antigens, such as ovalbumin or the soluble ectodomain of the envelope glycoprotein of human immunodeficiency virus (gp120), is low and often undetectable. On the other hand, it was found that LTB does exert very powerful adjuvant activity towards freely mixed large aggregated or particulate immunogens. These immunogens include influenza virus subunit antigen and keyhole limpet hemocyanin (KLH).

Accordingly, the present invention is concerned with a vaccine containing at least one particulate immunogen and an adjuvanting amount of LTB completely free of A subunit or toxic LT holotoxin.

As defined herein, "particulate" means any association of viral, bacterial, or fungal antigens characteristic of the respective micro-organisms. More in particular, the term "particulate immunogen" comprises aggregates, clusters, micelles, virosomes, rosettes, virus-like immunogen particles, and the like.

In the vaccine according to the present invention, in particular, LTB prepared from recombinant DNA technology can be utilised. The immunogen or immunogens may be derived from infective agents, such as viruses or bacteria.

Vaccines which apply to the above description were found not only to induce systemic immunoglobulin (e.g. IgG) against the immunogen upon mucosal (e.g. intra nasal) administration, but were also found to induce local secretion of IgA.

This latter property is particularly favourable for immunisation against diseases which are transmitted by mucosal infection with viruses (such as influenza virus, herpes virus, papilloma virus) or bacteria (like *Chlamydia*, pneumococs), or fungi.

A particular advantage of mucosal administration is the ease of vaccine application, which, furthermore, circumvents potential needlefobia with vaccinees receiving an intramuscular immunisation.

Although, for example in the case of influenza infection, high serum IgG titres are important for preventing systemic spread of the virus and protection of the lungs against infection, local S-IgA antibodies are crucial as a first line of defence for protection of the upper respiratory tract.

It has been reported that mucosal vaccination by i.n. administration of inactivated influenza virus in the absence of a mucosal adjuvant was not successful (Clancy: Drugs **50**: 587-594 [1995]; Katz et al.; J. Infect. Dis. **175**: 352-369 [1997]), probably because direct administration of an antigen to mucosal tissue will not result in an S-IgA response. Co-administration of a mucosal adjuvant seems to be a prerequisite to induce a local immune response against an immunogen. Remarkably, it was found that by i.n. immunisation according to the present invention the so-called common mucosal immune system is activated which results in secretion of S-IgA not only at the site op application (i.n.) but also in distant mucosal tissues (e.g. in the vaginal mucosal tissue).

Vaccines according to the present invention may contain immunogens of e.g. viral or bacterial origin, such as bacterial antigens, viral subunits (optionally inactivated) split viruses (optionally inactivated) inactivated viruses or bacteria, or attenuated (e.g. cold-adapted) live viruses, in a particulate form.

The LTB used according to the present invention is strictly free of toxic LTA or toxic holotoxin. Preferably, the LTB is prepared by recombinant DNA technology. Free of toxic LTA in the present context means strictly LTA-free.

In the vaccine according to the present invention the LTB can be used freely admixed with the particulate antigen - a covalent coupling between the antigen and the adjuvant can be established, however, is not needed to attain adequate adjuvant effect.

Apart from LTB and one or more immunogens the vaccine according to the present invention may contain an aqueous solvent, in particular a buffer, more in particular PBS (phosphate-buffered saline) as well as a stabiliser (e.g. PEG or methyl cellulose), and or glucose.

The components of the vaccine according to the present invention may be freeze dried or in a liquid form.

The vaccine according to the present invention may be present e.g. in bulk, or in an ampoule, or in a syringe, or in a nebuliser.

The vaccine according to the present invention may be administered by subcutaneous, or intramuscular, or intra-bronchial, or intra-nasal or intra-vaginal application or per os.

EXAMPLE 1

PREPARATION OF RECOMBINANT LTB AND INFLUENZA SUBUNIT ANTIGEN

RECOMBINANT LTB

Recombinant LTB genes and recombinant LTB molecules, as mentioned in the present invention, may be derived from genes encoding LT-I molecules from e.g. a porcine or a human source. The porcine LT (pLT) gene was subcloned in the pUC18 vector (Vieira and Messing: Gene **19**: 259-268 [1982]) using PCR techniques (DeHaan et al.: Vaccine **14**: 260-266 [1996]). The EWD299 vector, originally described by Dallas et al. (J. Bacteriol. **139**: 850-858 [1979]) was used as a template in the PCR reaction. The primary pLT sequence of this construct was found to be exactly in accordance with the primary pLT sequence as submitted in the EMBL sequence databank, as verified by DNA sequencing. From the pUC18-pLT construct,

the pLTB gene was subcloned in the pROFIT expression vector, which contains a temperature inducible λPR promoter(van der Linden et al.: Eur. J. Biochem. **204**: 197-202 [1992]).

E. coli MC1061 was used as a host strain for the pROFIT plasmid constructs. Bacteria were grown on Luria-Bertani medium containing 50 μg of kanamycin per ml. Induction of pLTB expression was obtained by raising the culture temperature of log-phase MC1061 cultures harboring the pROFIT-LTB vector from 28 to 42 degrees Celsius as described by De Haan et al. (*supra*).

pTLTB, a pKK-derived expression vector (Pharmacia Ltd.) encoding human LTB (i.e. an LTB gene derived from an LT gene isolated from an E. coli bacterium enterotoxigenic in humans) was obtained from Tamura and co-workers. DNA sequencing revealed 3 amino acid substitutions in the mature human LTB (hLTB) compared to pLTB (Thr4 to Ser, Glu46 to Ala, and Lys102 to Glu). *E. coli* strain JM101 was used as a host for pTLTB. Bacteria were grown on LB medium containing 100 μg of ampicillin per ml. Induction of hLTB expression was obtained by addition of IPTG to log-phase cultures of JM101 harboring pTLTB to a final concentration of 5 mM.

For purification of pLTB and hLTB, overexpressing bacteria were harvested, and then lysed by sonication. Subsequently, cell debris was removed by ultracentrifugation. Crude cell extracts containg recombinant pLTB or hLTB were then applied to an immobilised D-galactose (Pierce) column. After extensive washing, recombinant purified pLTB or hLTB were obtained by elution with D-galactose as previously described by Uesaka et al. (Microb. Path. **16**: 71-76 [1994]). Both recombinant pLTB and hLTB were found to retain optimal GM1-binding properties in a GM1 capture ELISA, as described previously (DeHaan et al.: Vaccine **14**: 260-266 [1996]). Column fractions containing purified protein were pooled, dialysed against PBS and stored at 4°C.

INFLUENZA SUBUNIT ANTIGEN

The influenza subunit antigen was prepared from B/Harbin/7/94 virus (B/Harbin) or A/Johannesburg/33/94 (A/Johannesburg) grown on embryonated chicken eggs according to the method described by Bachmayer et al. (Patent specification

GB 1 498 261 of January 18, 1978) and by Chaloupka et al. (Eur. J. Microbiol. Infect. Dis. **15**: 121-127 [1996]). This method comprises the steps of treatment of the formaldehyde-inactivated viruses with a suitable cationic detergent, separation of the released antigens (hemagglutinin and neuraminidase) from the virus residual core. This method leads to particulate, i.e. micelle-like exposition of the antigens after removal of the detergent.

The potency of the subunit antigen preparations, expressed as μg per ml, was determined in a single-radial diffusion test according to Wood et al. (J. Biol. Stand. **5**: 237-241 [1977]).

EXAMPLE 2

SYSTEMIC ANTIBODY RESPONSE TO INFLUENZA SUBUNIT VACCINE

Groups of four mice were immunised i.n. without anaesthesia with 5 μg of influenza subunit antigen derived from either B/Harbin or A/Johannesburg virus prepared according to the method described in EXAMPLE 1. The antigen was given either alone (HA) or together with 2.0 μg of pLTB (pLTB), in all cases in a volume of 20 μl on days 0, 7 and 14. Control mice received the same volume of PBS. Mice were sacrificed on day 28. Serum IgG antibody response was determined in a direct ELISA.

Figure 1 shows the observed serum IgG antibody responses against HA B/Harbin (solid bars) and HA A/Johannesburg (open bars).

Nasal administration of the subunit antigen without adjuvant gave a poor systemic antibody response, whereas supplementation of the subunit antigen with pLTB enhanced the serum antibody response by more than two orders of magnitude. Differences between the responses of mice immunised with B/Harbin and A/Johannesburg were not significant.

These results show that non-toxic pLTB is a powerful adjuvant capable of inducing high systemic antibody responses towards i.n. administered influenza subunit antigen.

EXAMPLE 3

COMPARISON OF SYSTEMIC ANTIBODY RESPONSES WITH HUMAN AND PORCINE LTB

Groups of 4 mice were immunised i.n. without anaesthesia with 5 μg of influenza subunit antigen derived from B/Harbin influenza virus prepared according to the method described in EXAMPLE 1.

The antigen was given either alone (NONE) or together with either 2.0 μg of pLTB (pLTB) or 2.0 μg of hLTB (hLTB), in all cases in a volume of 20 μl on days 0, 7 and 14. Control animals received PBS. Mice were sacrificed on day 21. Serum IgG antibody response was determined in a direct ELISA on day 21.

Figure 2 shows the observed serum IgG antibody response against HA B/Harbin. Nasal administration of subunit antigen without adjuvant again gave a poor systemic antibody responses, whereas supplementation of subunit antigen with pLTB and with hLTB enhanced the serum antibody response to the same extent by more than two orders of magnitude. The observed differences between pLTB and hLTB treated animals were non-significant.

EXAMPLE 4

INDUCTION OF LOCAL MUCOSAL ANTIBODY RESPONSE TO INFLUENZA SUBUNIT VACCINE

In order to investigate the ability of pLTB to evoke influenza HA-specific S-IgA responses, nasal washes of the mice from EXAMPLE 2 were analysed for the presence of influenza -specific IgA antibodies. Nasal washes were obtained by flushing 0.5 ml of PBS retrograde via the nasopharynx to the upper part of the trachea, flushing back, and collecting the lavage fluid at the nostrils.

The results are shown in Figure 3.

The data show that recombinant pLTB induced strong local S-IgA responses against HA. The two different influenza subunit antigens gave similar results.

EXAMPLE 5

COMPARISON OF MUCOSAL ANTIBODY RESPONSES WITH HUMAN AND PORCINE LTB

In order to compare the capacities of pLTB and hLTB to enhance nasal HA-specific antibody responses, nasal washes of the mice from EXAMPLE 3 were taken as described above and analyzed for the presence of HA-specific S-IgA on day 21. Figure 4 shows that both pLTB and hLTB induced brisk nasal HA-specific antibody responses. Moreover, the responses obtained with pLTB and hLTB were comparable in magnitude, demonstrating that both molecules have comparable adjuvant properties.

EXAMPLE 6

INDUCTION OF GENITAL MUCOSAL ANTIBODY RESPONSE TO INFLUENZA SUBUNIT VACCINE APPLIED I.N.

In order to investigate the ability of recombinant pLTB to evoke influenza HA-specific S-IgA responses at mucosal sites other than the site of administration, the induction of influenza-specific S-IgA antibodies in the genital tract after i.n. immunisation in the mice from EXAMPLE 2 was investigated. Lavages of the urogenital tract were conducted by introducing and withdrawing a 100 µl volume of PBS ten times into the vagina using a pipette tip. Mucosal washes were stored at 4 °C until determination of their IgA content by ELISA.The results are shown in Figure 5.
The results show that pLTB proved effective in inducing S-IgA responses at this distant mucosal site. Both B/Harbin and A/Johannesburg antigen responded equally well.

EXAMPLE 7

KINETICS OF IgG RESPONSE

Four groups of eight female BALB/c mice (6-8 weeks) each were treated as follows

Control treated with PBS without antigen. 20 µl i.n. without anaesthesia on days 0, 7 and 14

pLTB 5 µg HA and 2.0 µg recombinant pLTB in 20 µl applied i.n. without anaesthesia on days 0, 7 and 14

HA s.c. 5 µg HA in 100 µl applied s.c. without anaesthesia on day 0

Conv. convalescent mice, i.e. mice infected with 10^8 infective units of PR8 virus, in 20 µl applied i.n. without anaesthesia on day 0

From four mice of each group blood samples were taken from the tail veins on day 6, 13 and 20. Furthermore , on day 28 all mice were sacrificed and bled. In each sample serum IgG was measured by ELISA.

The results are shown in Figure 6. The bars (from left to right) for each of the vaccination regimens represent the IgG titres on days 6, 13, 20 and 28, respectively. These results show that after i.n. vaccination with HA/pLTB the IgG induction is of at least the same magnitude as after s.c. vaccination with HA alone, or as in convalescent mice.

EXAMPLE 8

NASAL AND LUNG MUCOSAL ANTIBODIES

The same mice which were studied in EXAMPLE 7 underwent mucosal lavages of the nasal cavity and the urogenital tract after being sacrificed on day 28 as described above.

The results are summarised in Figure 8. The hatched bars represent the data from nasal washes and the open bars show the data from vaginal washes.

The results indicate that the titre of the first line of defence antibodies (S-IgA) upon i.n. vaccination with HA/pLTB is of at least the same magnitude as the S-IgA titre in

convalescent mice whereas the (classical) s.c. vaccination with HA does not lead to a detectable mucosal IgA titre.

EXAMPLE 9

PROTECTION OF VACCINATED MICE AGAINST CHALLENGE

Four mice of each of the groups of EXAMPLE 7 were infected on day 28 with $5x10^6$ infective units of PR8 virus i.n. in 20 µl without anaesthesia.
Three days post-challenge virus load was determined in nose and lungs.

Virus titration in nose and lung homogenates was carried out on MDCK cells which were cultured on EPISERF (Life Technologies, PAISLY, Scotland) in microtitration plates by two-step dilutions, and by subsequent endpoint determination using haemagglutination with guinea pig erythocytes.

The results are summarised in Figure 7. The hatched bars represent the virus titres in the nose and the open bars are for the lungs. The virus titres in the lungs for convalescent mice and upon vaccination with pLTB were insignificant. Hence, these data show that by using pLTB as a mucosal adjuvant, protection against influenza infection is complete.

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CLAIMS

1. Vaccine for mucosal administration containing at least one particulate immunogen and an adjuvanting amount of B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli.*, completely free of A subunit or toxic LT holotoxin.

2. Vaccine according to claim 1, wherein the LTB is prepared by recombinant DNA methods.

3. Vaccine according to claim 1-2, wherein viral or bacterial or fungal antigens are used as an immunogen.

4. Vaccine according to claim 1-3, wherein the immunogen provides immunisation against a disease which is transmitted by mucosal infection.

5. Vaccine according to claim 4, wherein influenza antigens are used as an immunogen.

6. Method for the induction of a systemic immunoglobulin response against an immunogen by mucosal administration of said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli*, completely free of A subunit or toxic LT holotoxin.

7. Method for the induction of a common mucosal immune response against an immunogen by mucosal administration of said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli*, completely free of A subunit or toxic LT holotoxin.

8. Use of the B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli* , completely free of A subunit or toxic LT holotoxin in the preparation of a vaccine comprising a particulate immunogen and an adjuvanting amount of said LTB suitable for the induction of a systemic immunoglobulin response against said immunogen in an individual upon mucosal administration.

9. Use of the B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli* , completely free of A subunit or toxic LT holotoxin in the preparation of a vaccine comprising a particulate immunogen and an adjuvanting amount of said LTB suitable for the induction of a common mucosal immune response against said immunogen in an individual upon local mucosal administration.

FIGURE 1

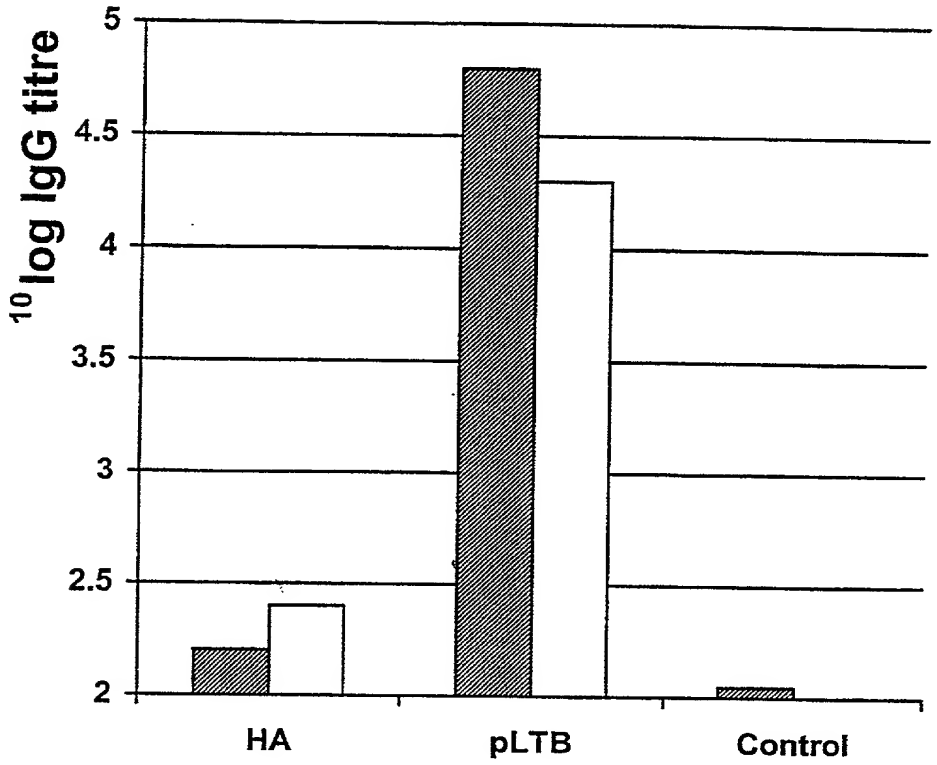
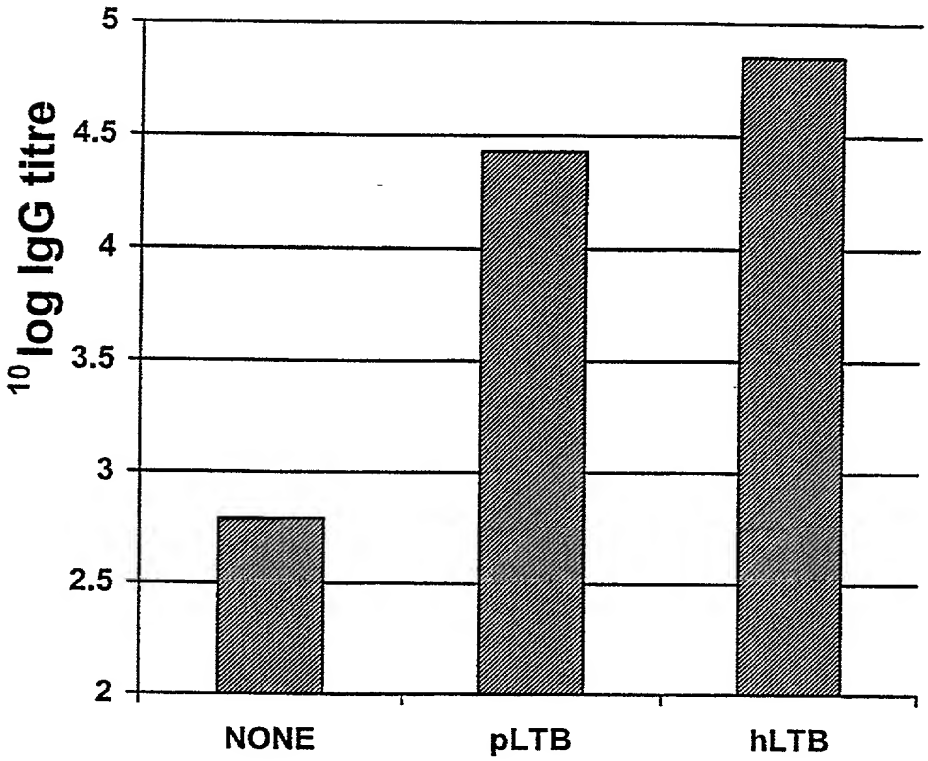


FIGURE 2



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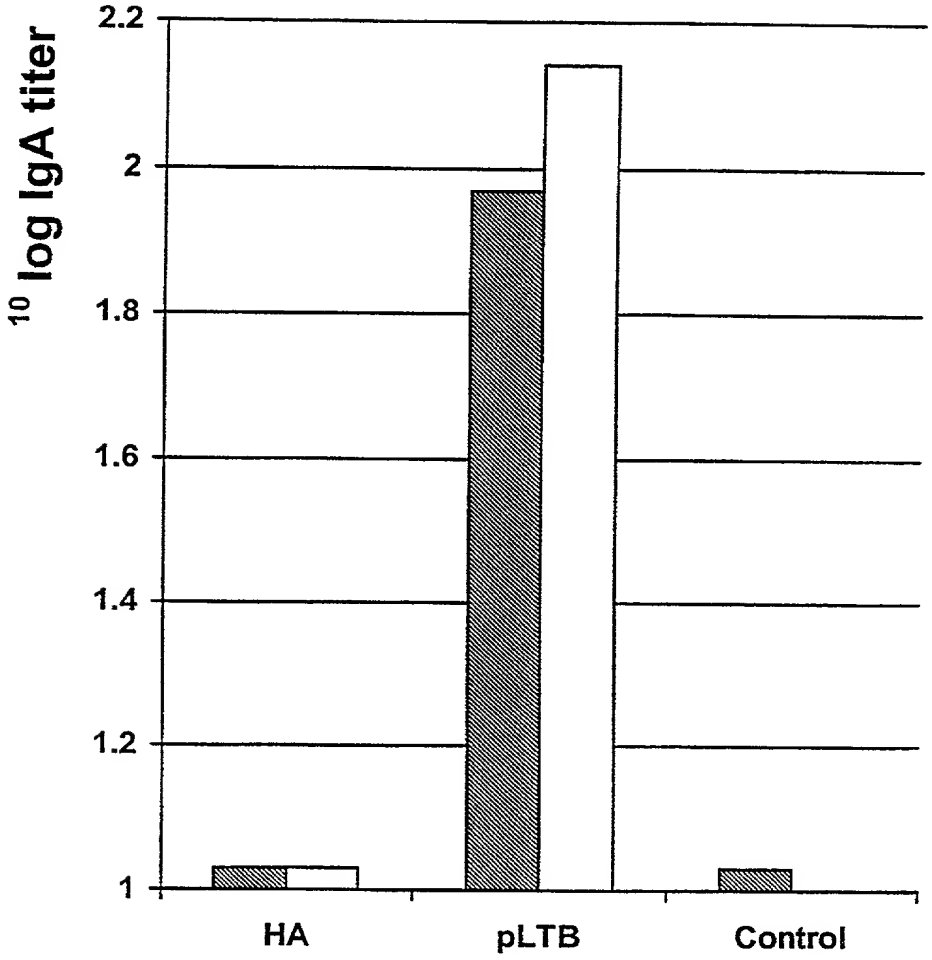


FIGURE 4

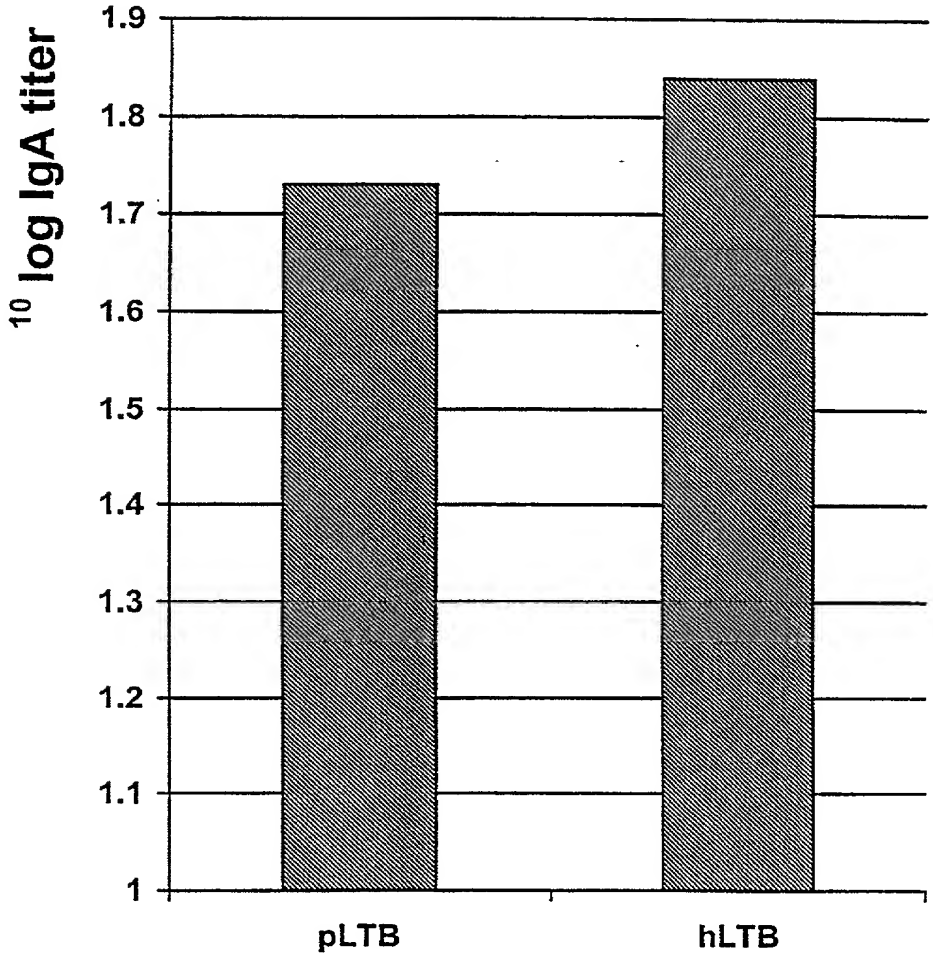


FIGURE 5

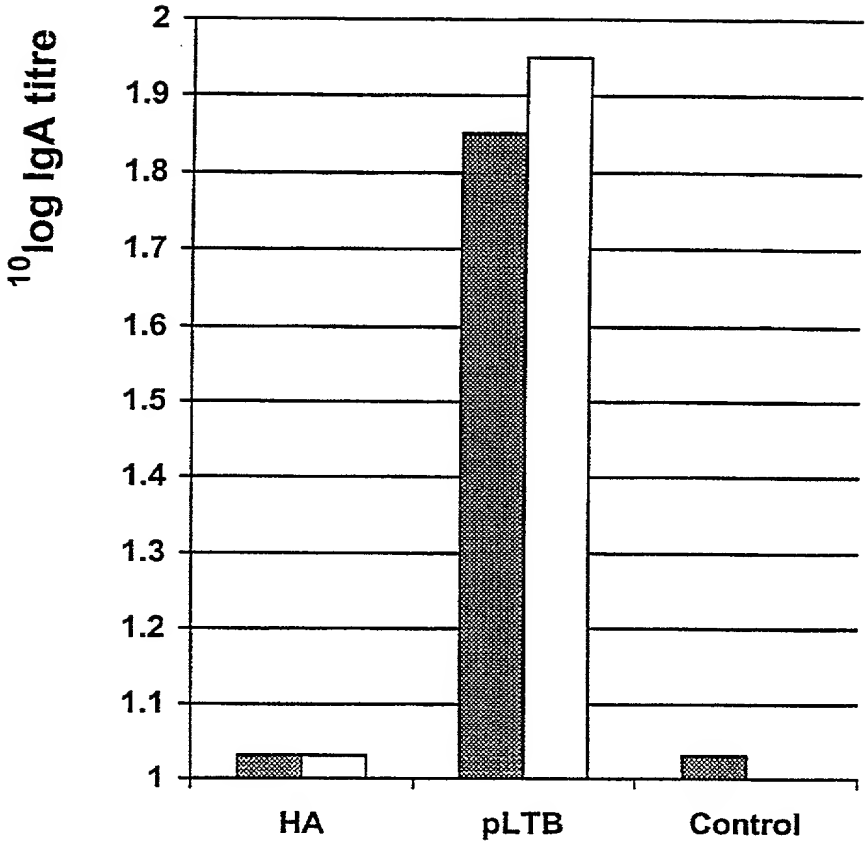


FIGURE 6

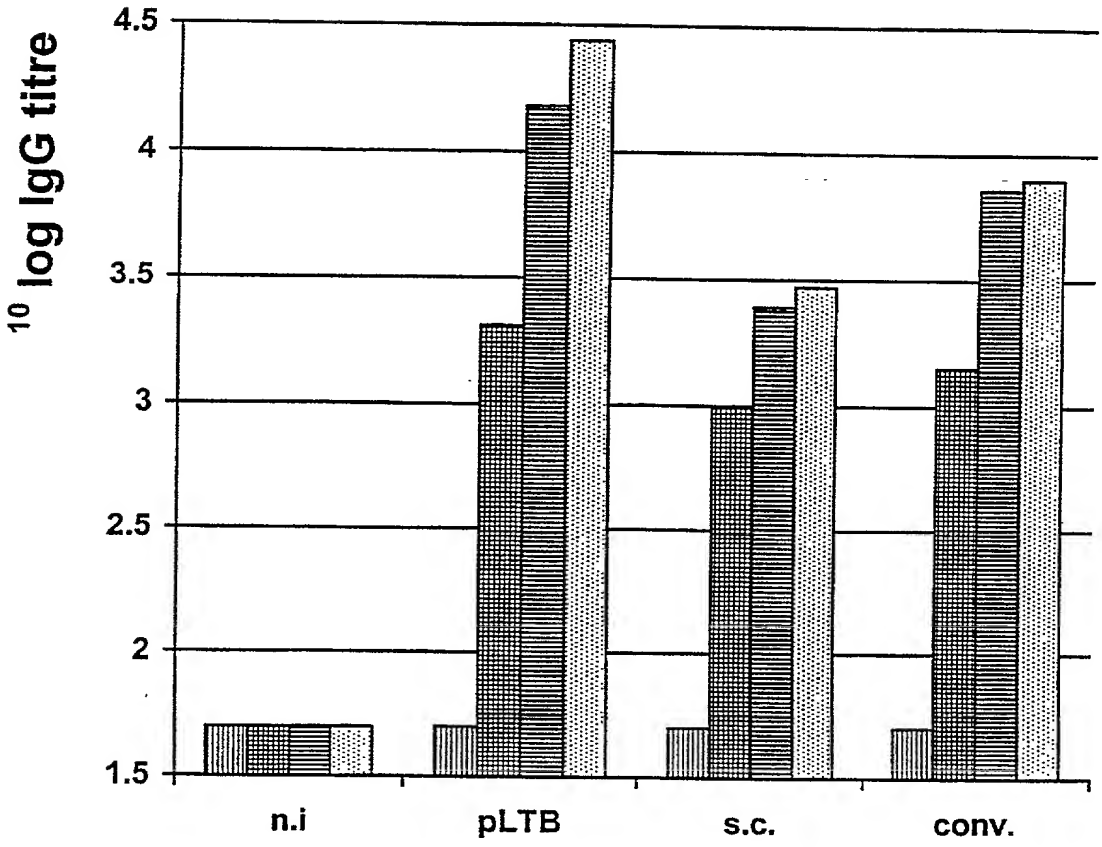


FIGURE 7

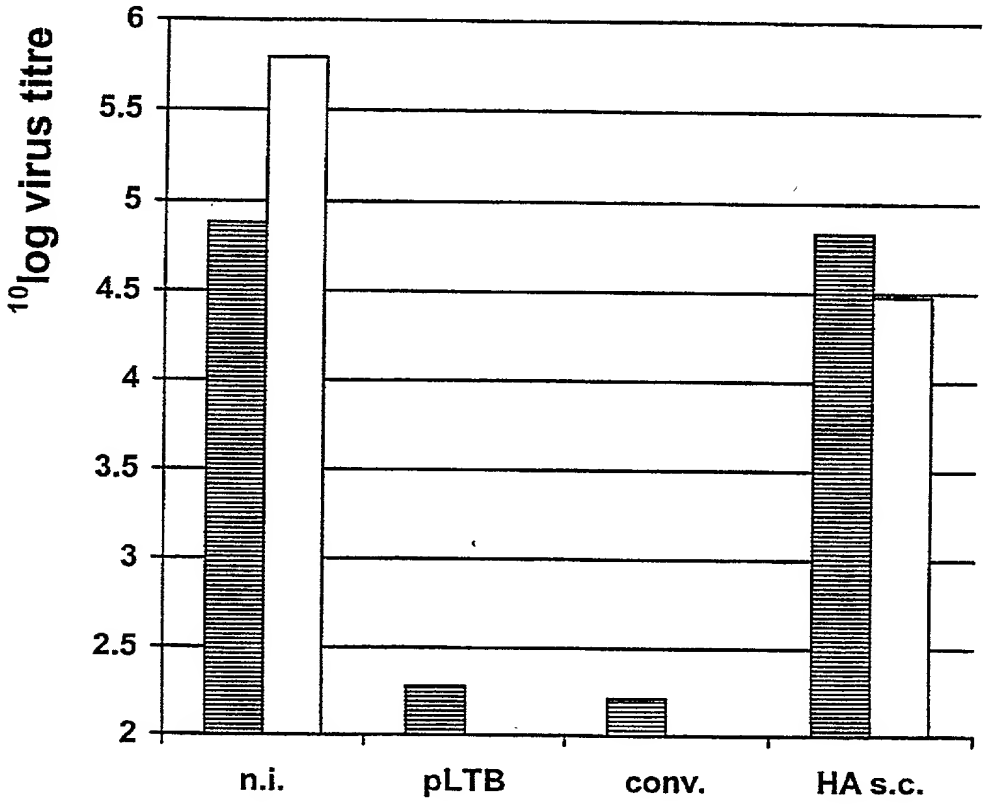
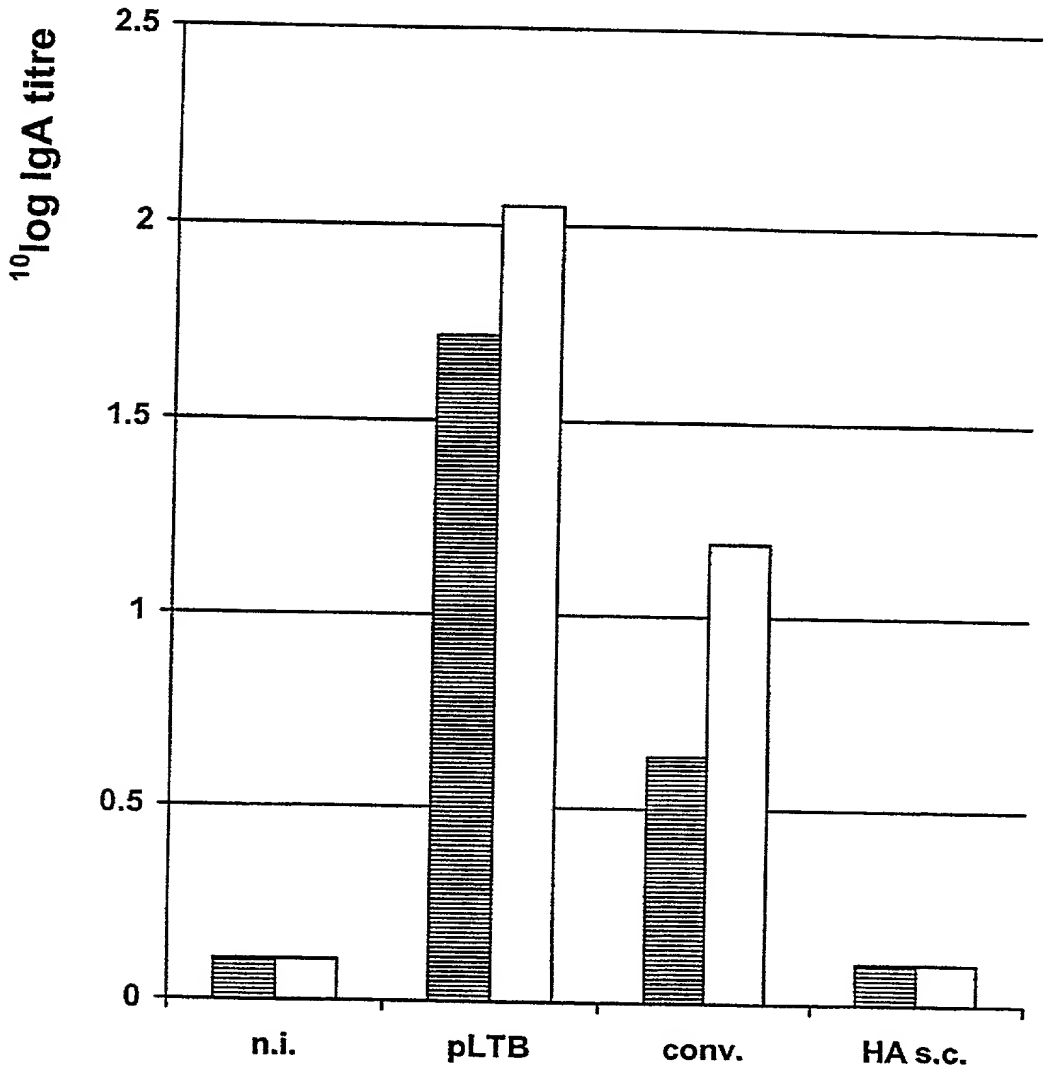


FIGURE 8



Attorney Docket No.: DIR 0549 US
Declaration/Power of Attorney
Worldwide Rights

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I/We hereby declare that: my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: Vaccines with an LTB adjuvant the specification of which ☜ is attached and/or ☜ was filed on _____ as United States Application Serial No. _____ or PCT International Application No. PCT/EP98/07553 and was amended on 28 September 1999 .

I/We hereby state that I/We have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I/We acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I/We hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application(s) designating at least one country other than the United States, listed below and have also identified below, any foreign application(s) for patent or inventor's certificate, or any PCT International application(s) having a filing date before that of the application(s) of which priority is claimed:

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. 119
EP (NL designated)	97203671.9	25 november 1997	× YES NO

I/We hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing

I/We hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application(s) and the national or PCT International filing date of this application:

Application Number	Date of Filing	Status (Patented, Pending, Abandoned)

I/We hereby appoint the following attorney and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. **FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.,** Douglas B. Henderson, Reg. No. 20,291; Ford F. Farabow, Jr., Reg. No. 20,630; Arthur S. Garrett, Reg. No. 20,338; Donald R. Dunner, Reg. No. 19,073; Brian G. Brunsvold, Reg. No. 22,593; Tipton D. Jennings, IV, Reg. No. 20,645; Jerry D. Voight, Reg. No. 23,020; Laurence R. Hefter, Reg. No. 20,827; Kenneth E. Payne, Reg. No. 23,098; Herbert H. Mintz, Reg. No. 26,691; C. Larry O'Rourke, Reg. No. 26,014; Albert J. Santorelli, Reg. No. 22,610; Michael C. Elmer, Reg. No. 25,857; Richard H. Smith, Reg. No. 20,609; Stephen L. Peterson, Reg. No. 26,325; John M. Romary, Reg. No. 26,331; Bruce C. Zotter, Reg. No. 27,680; Dennis P. O'Reilley, Reg. No. 27,932; Allen M. Sokal, Reg. No. 26,695; Robert D. Bajefsky, Reg. No. 25,387; Richard L. Stroup, Reg. No. 28,478; David W. Hill, Reg. No. 28,220; Thomas L. Irving, Reg. No. 28,619; Charles E. Lipsey, Reg. No. 28,165; Thomas W. Winland, Reg. No. 27,605; Basil J. Lewris, Reg. No. 28,818; Martin I. Fuchs, Reg. No. 28,508; E. Robert Yoches, Reg. No. 30,120; Barry W. Graham, Reg. No. 29,924; Susan Haberman Griffen, Reg. No. 30,907; Richard B. Racine, Reg. No. 30,415; Thomas H. Jenkins, Reg. No. 30,857; Robert E. Converse, Jr., Reg. No. 27,432; Clair X. Mullen, Jr., Reg. No. 20,348; Christopher P. Foley, Reg. No. 31,354; John C. Paul, Reg. No. 30,413; Roger D. Taylor, Reg. No. 28,992; David M. Kelly, Reg. No. 30,953; Kenneth J. Meyers, Reg. No. 25,146; Carol P. Einaudi, Reg. No. 32,220; Walter Y. Boyd, Jr., Reg. No. 31,738; Steven M. Anzalone, Reg. No. 32,095; Jean B. Fordis, Reg. No. 32,984; Barbara C. McCurdy, Reg. No. 32,120; James K. Hammond, Reg. No. 31,964; Richard V. Burgujian, Reg. No. 31,744; J. Michael Jakes, Reg. No. 32,824; Dirk D. Thomas, Reg. No. 32,600; Thomas W. Banks, Reg. No. 32,719; Christopher P. Isaac, Reg. No. 32,616; Bryan C. Diner, Reg. No. 32,409; M. Paul Barker, Reg. No. 32,013; Andrew Chanho Sonu, Reg. No. 33,457; David S. Forman, Reg. No. 33,694; Vincent P. Kovalick, Reg. No. 32,867; James W. Edmondson, Reg. No. 33,871; Michael R. McGurk, Reg. No. 32,045; Joann M. Neth, Reg. No. 36,363; Gerson S. Panitch, Reg. No. 33,751; Cheri M. Taylor, Reg. No. 33,216; Charles E. Van Horn, Reg. No. 40,266; Linda A. Wadler, Reg. No. 33,218; Jeffrey A. Berkowitz, Reg. No. 36,743; Michael R. Kelly, Reg. No. 33,921; and James B. Monroe, Reg. No. 33,971; and _____.

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I/We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

IN TESTIMONY WHEREOF, I/We have hereunto set our hands.

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Residence: Weesp, The Netherlands (of the Deceased at Time of Death) Weesp, The Netherlands (Executor) NLX		Citizenship: Dutch (Deceased as well as Executor)
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